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**APPLICATION NUMBER:** *60/022,684*
**FILING DATE:** *July 26, 1996*

PRIORITY DOCUMENT

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# SUPPLEMENTAL PROVISIONAL APPLICATION COVER SHEET
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| Docket Number | 850103.401P1 | Type a plus sign (+) inside this box → | + |
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**TITLE OF THE INVENTION** (280 characters max)

CYCLIC DECAPEPTIDE ANTIBIOTICS

**CORRESPONDENCE ADDRESS**

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Respectfully submitted,

SIGNATURE _____ Date 10/9/96

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☐ Additional inventors are being named on separately numbered sheets attached hereto.

DWP/401

PATENT

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David W. Parker

THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Raymond J. Andersen et al.

Application No. : 60/022,684

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Docket No. : 850103.401P1

Box Provisional Application
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Date

David W. Parker

THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Raymond J. Andersen et al.

Application No. : 60/022,684

Filed : July 26, 1996

For : CYCLIC DECAPEPTIDE ANTIBIOTICS

Docket No. : 850103.401P1

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DWP\253

Descripti n

# CYCLIC DECAPEPTIDE ANTIBIOTICS

## Technical Field

The present invention relates to novel cyclic peptide compounds with potent activity against antibiotic-resistant human pathogens.

## Background of the Invention

Methicillin-resistant strains of *Staphylococcus aureus* (MRSA) cause infections that are refractory to standard anti-staphylococci antibiotics, and in many cases vancomycin is the antibiotic of last resort. Consequently, it is of great concern that vancomycin-resistant strains of MRSA may develop.

Infections due to enterococci have been difficult to treat for many years because these organisms are intrinsically resistant to many antibiotics. Ampicillin has been the mainstay for treatment of uncomplicated enterococcal infections, but many strains have now become resistant to ampicillin. Vancomycin is again the only effective treatment for these ampicillin-resistant enterococcal infections. In the past few years, vancomycin-resistant enterococcal strains (VRE) have begun to appear and they are rapidly spreading across North America. There are no effective antibiotics currently available for such organisms and the recent report of an outbreak of VRE with a 73% mortality rate has highlighted the seriousness of the situation. *See* Edmond, M.B. et al., *Clinical Infectious Diseases 20*:1126-33, 1995.

One area where new drugs are desperately needed is in the treatment of antibiotic-resistant strains of gram positive human pathogens. The present invention is directed to fulfilling this need, and provides related advantages as described herein.

Summary of the Invention

The invention is directed to isolated cyclic decapeptides of the formulae: cyclo [L-Val-L-Orn-L-Leu-D-Tyr-L-Pro-L-Phe-D-Phe-L-Asn-L-Asp-L-Tyr];

cyclo [L-Val-L-Orn-L-Leu-D-Tyr-L-Pro-L-Phe-D-Phe-L-Asn-L-Asp-L-Trp]; and

cyclo [L-Val-L-Orn-L-Leu-D-Tyr-L-Pro-L-Trp-D-Phe-L-Asn-L-Asp-L-Trp].

The invention is directed to the above-identified cyclic decapeptides in an isolated, *i.e.*, substantially purified form, preferably in a quantity of more than about 1 gram, more preferably in a quantity of more than about 10 grams, still more preferably in a quantity of more than about 100 grams, and yet still more preferably in a quantity of more than about 1 kilogram. A substantially purified form is a composition wherein the above-listed cyclic decapeptides constitute at least about 1 weight percent of the composition, preferably at least about 10 weight percent, more preferably at least about 30 weight percent, still more preferably at least about 50 weight percent, yet still more preferably at least about 70 weight percent, and yet still more preferably at least about 95 weight percent, and most preferably at least about 99 weight percent.

The invention is also directed to the above-identified cyclic decapeptides in a pharmaceutical composition. A pharmaceutical composition of the invention may not necessarily contain the cyclic decapeptide in a substantially purified form because the composition may contain diluent and/or other materials commonly found in pharmaceutical compositions.

The invention is also directed to a method of treating bacterial infection, comprising administering to a patient having a bacterial infection, an amount of the above-identified cyclic decapeptides effective to relieve symptoms associated with or due to the bacterial infection.

Detailed Description of the Invention

The invention is directed to isolated cyclic decapeptides having one of formulas (1), (2) or (3) shown below, and are named Loloatin A, Loloatin B and Loloatin C, respectively.

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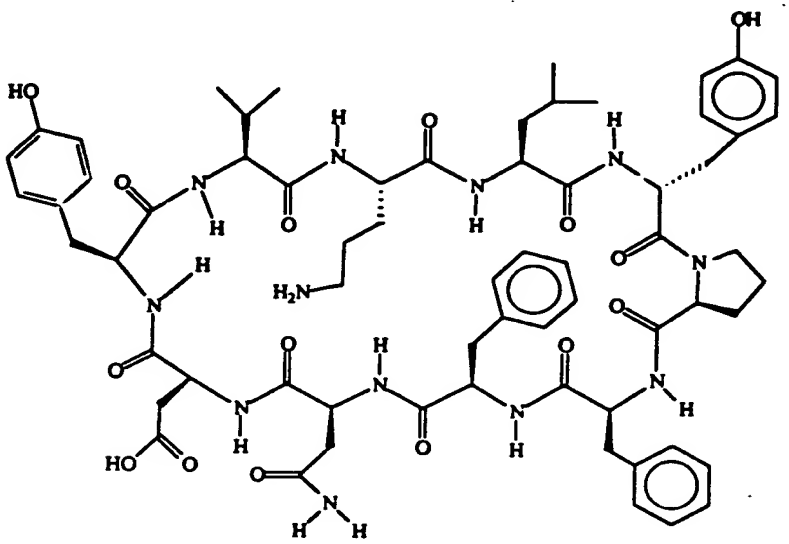
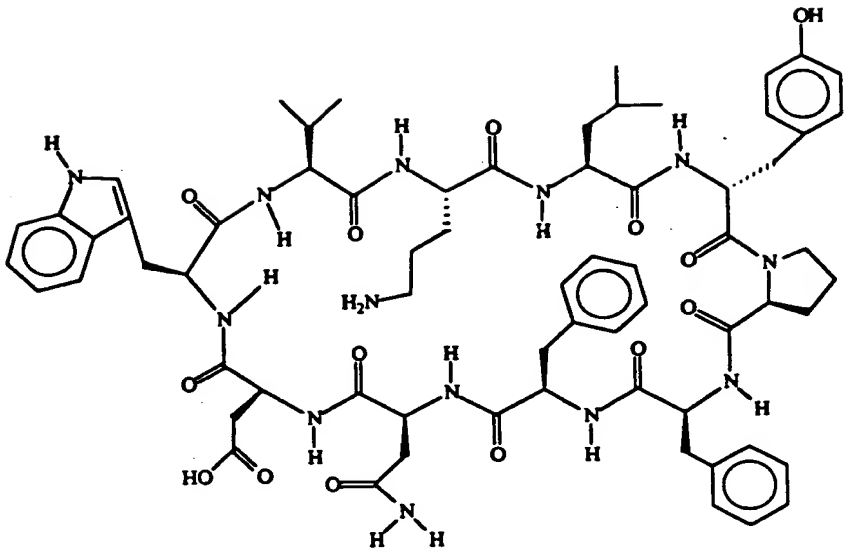


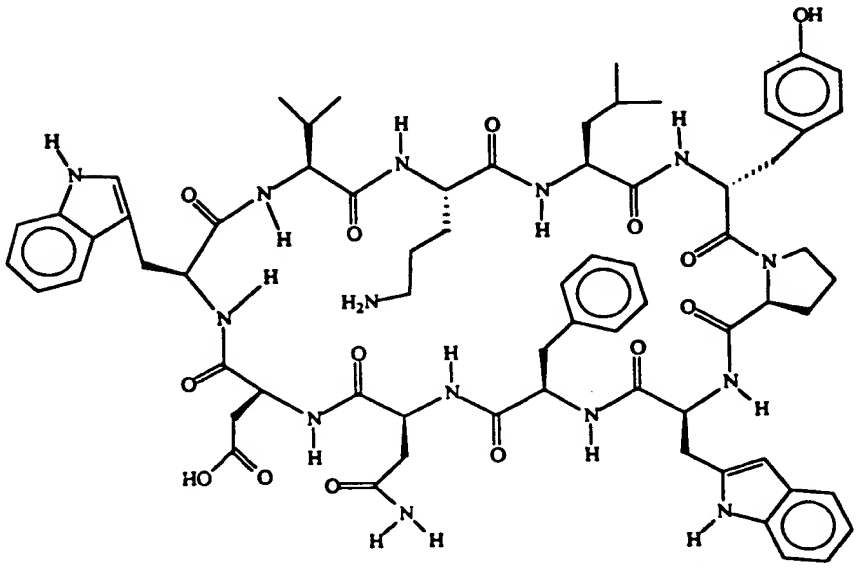
(1)

Loloatin A



(2)

Loloatin B



(3)

Loloatin C

Written in different terms, Loloatin A, B and C have the amino acid sequences set forth in Table A below, where the first listed amino acid is joined through a peptide bond to the last listed amino acid so as to form a cyclic structure.

TABLE A

| Loloatin A | Loloatin B | Loloatin C |
|---|---|---|
| L-Val | L-Val | L-Val |
| L-Orn | L-Orn | L-Orn |
| L-Leu | L-Leu | L-Leu |
| D-Tyr | D-Tyr | D-Tyr |
| L-Pro | L-Pro | L-Pro |
| L-Phe | L-Phe | L-Trp |
| D-Phe | D-Phe | D-Phe |
| L-Asn | L-Asn | L-Asn |
| L-Asp | L-Asp | L-Asp |
| L-Tyr | L-Trp | L-Trp |

For ease of reference, the compounds of formulas (1), (2) and (3), *i.e.*, Loloatin A, B and C, will collectively be referred to herein as the compounds of Formula (A) or the cyclic decapeptides (or compounds) of the invention.

The cyclic decapeptides of the present invention may be prepared *in vitro*, using solid phase or solution peptide synthesis techniques, or may be prepared *in vivo*, from microorganism ATCC 55797. Solution phase techniques as set forth in K. Okamato, K. et al. *Bull. Chem. Soc. Jpn.* 50:231-236 (1977), Ohno, M. et al. *J. Am. Chem. Soc.* 88(2):376-377 and Kosui, N. et al. *Int. J. Peptide Protein Res.* 18:127-134 (1981) may be modified to prepare the cyclic decapeptides of the present invention, merely by appropriate substitution of the suitably protected amino acids. Ösapay, G.; Profit, A.; Taylor, J.W., "Synthesis of Tyrocidine A: Use of Oxime Resin for Peptide Chain Assembly and Cyclization," *Tetrahedron Letters 131*(43):6121-6124 (1990) describes a synthetic scheme (termed PCOR for Peptide Cyclization on Oxime Resin) using a solid support, which can be modified to prepare the cyclic decapeptides of the present invention. The cyclic decapeptides of the invention may also be isolated from microorganism ATCC 55797.

The cyclic decapeptides of the invention have utility as antibiotics, and may be used and administered in a manner analogous to antibiotics known in the art, to provide the beneficial effects desired of antibiotics. Preferably, the use is in the veterinary or, more preferably, the pharmaceutical field. Thus, the invention extends to the use of any compound of Formula (A) for the manufacture of a medicament for use in therapy. The invention further provides the use of any compound of Formula (A) for the manufacture of a medicament for use in the treatment of methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecalis*, and *Streptococcus pneumoniae* infections in a mammal. The cyclic decapeptides of the invention may be used against gram negative and gram positive bacteria.

In using a compound of Formula (A), the compound is preferably administered to a patient in a pharmaceutical or veterinary composition comprising also a pharmaceutically or veterinarily acceptable carrier, and optionally, one or more other biologically active ingredients. Such compositions may be in any form used for oral, topical, vaginal, parenteral, rectal and inhalatory application. The compositions may be provided in discrete dose units. The carriers may be particulate, with compositions being, for example, tablets or powders, or liquid, with the compositions being, for example, oral syrups or injectable liquids, or gaseous, for inhalatory application.

For oral administration, an excipient and/or binder may be present. Examples are sucrose, kaolin, glycerin, starch dextrins, sodium alginate, carboxymethylcellulose and ethyl cellulose. Coloring and/or flavoring agents may be present. A coating shell may be employed. For rectal administration oleaginous bases may be employed, for example, lanolin or cocoa butter. For an injectable formulation, buffers, stabilizers and isotonic agents may be included.

It will be evident to those of ordinary skill in the art that the optimal dosage of the compounds of Formula (A) may depend on the weight and physical condition of the patient; on the severity and longevity of the illness; and on the particular form of the active ingredient, the manner of administration and the composition employed.

It is to be understood that use of a compound of Formula (A) in chemotherapy can involve such a compound being bound to an agent, for example, a

monoclonal or polyclonal antibody, a protein or a liposome, which assist the delivery of said compound to the site of infection.

Therefore, the invention relates further to a pharmaceutical or veterinary composition comprising an effective amount of compound of Formula (A) in association with a carrier.

In a further embodiment, the present invention provides a method for the treatment of a patient afflicted with a bacterial infection comprising the administration thereto of a therapeutically effective amount of a compound of Formula (A).

The term "therapeutically effective amount" refers to an amount which is effective, upon single or multiple dose administration to the patient, in providing relief of symptoms associated with bacterial infections. As used herein, "relief of symptoms" of a bacterial infection refers to a decrease in severity over that expected in the absence of treatment and does not necessarily indicate a total elimination or cure of the infection or condition caused thereby. In determining the therapeutically effective amount or dose, a number of factors are considered by the attending diagnostician, including, but not limited to: the species of mammal; its size, age, and general health; the specific infection involved; the degree of or involvement or the severity of the infection or condition arising therefrom; the response of the individual patient; the particular compound administered; the mode of administration; the bioavailability characteristics of the preparation administered; the dose regimen selected; the use of concomitant medication; and other relevant circumstances.

A therapeutically effective amount of a compound of Formula (A) is expected to vary from about 0.1 milligram per kilogram of body weight per day (mg/kg/day) to about 100 mg/kg/day. Preferred amounts are expected to vary from about 0.5 to about 10 mg/kg/day.

In effecting treatment of a patient afflicted with a condition described above, a compound of Formula (A) can be administered in any form or mode which makes the compound bioavailable in effective amounts, including oral, aerosol, and parenteral routes. For example, compounds of Formula (A) can be administered orally, by aerosolization, subcutaneously, intramuscularly, intravenously, transdermally, intranasally, rectally, topically, and the like. Oral or aerosol administration is generally

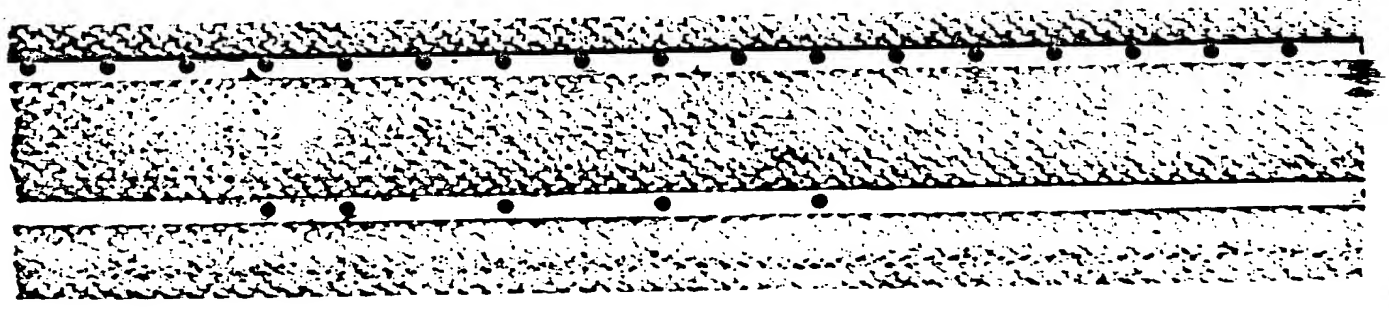
preferred. One skilled in the art of preparing formulations can readily select the proper form and mode of administration depending upon the particular characteristics of the compound selected, the condition to be treated, the stage of the condition, and other relevant circumstances. *See, e.g.*, Remington's Pharmaceutical Sciences, 18th Edition, Mack Publishing Co. (1990).

The compounds can be administered alone or in the form of a pharmaceutical composition in combination with pharmaceutically acceptable carriers or excipients, the proportion and nature of which are determined by the solubility and chemical properties of the compound selected, the chosen route of administration, and standard pharmaceutical practice.

In another embodiment, the present invention provides compositions comprising a compound of Formula (A) in admixture or otherwise in association with one or more inert carriers. These compositions are useful, for example, as assay standards, as convenient means of making bulk shipments, or as pharmaceutical compositions. An assayable amount of a compound Formula (A) is an amount which is readily measurable by standard assay procedures and techniques as are well known and appreciated by those skilled in the art. Assayable amounts of a compound Formula (A) will generally vary from about 0.001% to about 75% of the composition by weight. Inert carriers can be any material which does not degrade or otherwise covalently react with a compound of Formula (A). Examples of suitable inert carriers are water; aqueous buffers, such as those which are generally useful in High Performance Liquid Chromatography (HPLC) analysis; organic solvents, such as acetonitrile, ethyl acetate, hexane and the like; and pharmaceutically acceptable carriers or excipients.

More particularly, the present invention provides pharmaceutical compositions comprising a therapeutically effective amount of a compound of Formula (A) in admixture or otherwise in association with one or more pharmaceutically acceptable carriers or excipients.

The pharmaceutical compositions are prepared in a manner well known in the pharmaceutical art. The carrier or excipient may be a solid, semi-solid, or liquid material which can serve as a vehicle or medium for the active ingredient. Suitable carriers or excipients are well known in the art. The pharmaceutical composition may be



adapted for oral, parenteral, or topical use and may be administered to the patient in the form of tablets, capsules, suppositories, solution, suspensions, or the like.

The compounds of the present invention may be administered orally, for example, with an inert diluent or with an edible carrier. They may be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the compounds may be incorporated with excipients and used in the form of tablets, troches, capsules, elixirs, suspensions, syrups, wafers, chewing gums and the like. These preparations should contain at least 4% of the compound of the invention, the active ingredient, but may be varied depending upon the particular form and may conveniently be between 4% to about 70% of the weight of the unit. The amount of the compound present in compositions is such that a suitable dosage will be obtained. Preferred compositions and preparations according to the present invention are prepared so that an oral dosage unit form contains between 5.0-300 milligrams of a compound of the invention. The tablets, pills, capsules, troches and the like may also contain one or more of the following adjuvants: binders such as microcrystalline cellulose, gum tragacanth or gelatin; excipients such as starch or lactose, disintegrating agents such as alginic acid, Primogel, corn starch and the like; lubricants such as magnesium stearate or Sterotex; glidants such as colloidal silicon dioxide; and sweetening agents such as sucrose or saccharin may be added or a flavoring agent such as peppermint, methyl salicylate or orange flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier such as polyethylene glycol or a fatty oil. Other dosage unit forms may contain other various materials which modify the physical form of the dosage unit, for example, as coatings. Thus, tablets or pills may be coated with sugar, shellac, or other enteric coating agents. A syrup may contain, in addition to the present compounds, sucrose as a sweetening agent and certain preservatives, dyes and colorings and flavors. Materials used in preparing these various compositions should be pharmaceutically pure and non-toxic in the amounts used.

For the purpose of parenteral therapeutic administration, the compounds of the present invention may be incorporated into a solution or suspension. These preparations should contain at least 0.1% of a compound of the invention, but may be varied to be between 0.1 and about 50% of the weight thereof. The amount of the

inventive compound present in such compositions is such that a suitable d sage will be btained. Preferred compositions and preparations according to the present invention are prepared so that a parenteral dosage unit contains between 5.0 to 100 milligrams of the compound of the invention.

The compounds of Formula (A) of the present invention may also be administered by aerosol. The term aerosol is used to denote a variety of systems ranging from those of colloidal nature to systems consisting of pressurized packages. Delivery may be by a liquefied or compressed gas or by a suitable pump system which dispenses the active ingredients. Aerosols of compounds of Formula (A) may be delivered in single phase, bi-phasic, or tri-phasic systems in order to deliver the active ingredient. Delivery of the aerosol includes the necessary container, activators, valves, subcontainers, and the like. Preferred aerosols are able to be determined by one skilled in the art.

The compounds of Formula (A) of this invention may also be administered topically, and when done so the carrier may suitably comprise a solution, ointment or gel base. The base, for example, may comprise one or more of the following: petrolatum, lanolin, polyethylene glycols, bee wax, mineral oil, diluents such as water and alcohol, and emulsifiers and stabilizers. Topical formulations may contain a concentration of the Formula (A) compound of from about 0.1 to about 10% w/v (weight per unit volume).

The solutions or suspensions may also include one or more of the following adjuvants: sterile diluents such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerin, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl paraben; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

The cyclic decapeptides of the invention may be combined with one or more known antibiotics to provide a synergistic composition. In other words, a composition comprising a cyclic decapeptide of the invention and a known antibiotic

may have greater efficacy against bacteria than would be expected based on the individual efficacies of the cyclic decapeptide and the known antibiotic.

As used herein, the term "patient" refers to a warm-blooded animal such as a mammal which is afflicted with a particular inflammatory disease state. It is understood that guinea pigs, dogs, cats, rats, mice, horses, cattle, sheep, and humans are examples of animals within the scope of the meaning of the term.

The following examples are offered by way of illustration and not by way of limitation.

## EXAMPLES

Symbols and abbreviations used herein are in accordance with the recommendation of IUPAC-IUB Commissioner on Biochemical Nomenclature, *J. Biol. Chem.* 1971, 247, 977. Abbreviations: "Asn" refers to asparagine; "Asp" refers to aspartic acid, "bp" refers to boiling point; BOP = benzotriazol-1-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate; "BrZ" refers to bromobenzyloxycarbonyl; "Bzl" refers to benzyl; "°C" refers to degrees Celsius; CD = circular dichroism; "DCC" refers to N,N-dicyclohexylcarbodiimide; DCM = dichloromethane; DIEA = N,N-diisopropylethylamine; DMF = *N,N*-dimethylformamide; "DPPA" refers to diphenylphosphorylazide; FAB-MS = fast atom bombardment mass spectrometry; "HOBt" refers to 1-hydroxybenzotriazole; "g" refers to grams; "Leu" refers to leucine; "mL" refers to milliliters; "mm Hg" refers to millimeters of mercury; "mmol" refers to millimoles; "NMP" refers to *N*-methylpyrrolidinone; "Orn" refers to ornithine; "Phe" refers to phenylalanine; "Pro" refers to proline; "TEA" refers to triethylamine; TMSOTf = trimethylsilyl trifluoromethanesulfonate; "Tos" refers to *p*-toluenesulfonyl;. "Trp" refers to tryptophan; "Tyr" refers to tyrosine; "Val" refers to valine, Z = benzyloxycarbonyl; "µg" refers to micrograms; "µL" refers to microliters; and. "µM" refers to micromolar.

### A. PREPARATIVE EXAMPLES

The cyclic decapeptides of the present invention may be prepared *in vitro*, using solid phase or solution peptide synthesis techniques, or may be prepared *in vivo*,

from microorganism ATCC 55797. Solution phase techniques as set forth in K. Okamato, K. et al. *Bull. Chem. Soc. Jpn.* 50:231-236 (1977), Ohno, M. et al. *J. Am. Chem. Soc.* 88(2):376-377 and Kosui, N. et al. *Int. J. Peptide Protein Res.* 18:127-134 (1981) may be modified to prepare the cyclic decapeptides of the present invention, merely by appropriate substitution of the suitably protected amino acids.

The following examples present typical syntheses. These examples are understood to be illustrative only and are not intended to limit the scope of the present invention in any way.

EXAMPLE A1

Ösapay, G.; Profit, A.; Taylor, J.W., "Synthesis of Tyrocidine A: Use of Oxime Resin for Peptide Chain Assembly and Cyclization," *Tetrahedron Letters 131*(43):6121-6124 (1990) describes a synthetic scheme (termed PCOR for Peptide Cyclization on Oxime Resin) using a solid support, which can be modified to prepare the cyclic decapeptides of the present invention. Further details of the PCOR method may be found in Ösapay, G.; Bouvier, M ; Taylor, J.W., "Peptide Cyclization on Oxime Resin (PCOR)" in Techniques in Protein Chemistry II, (ed. Villafranca, J.J.). The following description illustrates the synthesis of Loloatin A.

The *p*-nitrobenzophenone oxime polymer described by DeGrado and Kaiser may be used as a solid support in preparing cyclic decapeptides of the present invention. See DeGrado, W.F.; Kaiser, E.T , *J. Org. Chem.* 45:1295-1300 (1980). For the preparation of, for example, cyclo[L-Val-L-Orn-L-Leu-D-Tyr-L-Pro-L-Phe-D-Phe-L-Asn-L-Asp-L-Tyr] (Loloatin A) the starting compound Boc-Leu-resin is the same as described in the Ösapay article, and the excess oxime groups may be capped by acetylation as described therein. The peptide chain may be assembled by consecutive addition of the following $N^{\alpha}$-Boc-amino acids, which have the L-configuration unless otherwise noted: BocOrn(Z)OH, BocValOH, BocTyr(2,6-$Cl_2$-Bzl)OH, BocAsp(β-Bzl)OH, BocAsnOH, Boc-D-PheOH, BocPheOH, BocProOH and BocTyr(2,6-$Cl_2$-Bzl)OH, all according to the BOP peptide coupling procedure of Fournier, A.; Wang, C.T.; Felix, A.M., *Int. J. Pept., Prot. Res.*, 31:86-97 (1988).

Boc protecting groups may be removed by treatment with 25% TFA/DCM solution for 30 minutes. After the appropriate washing steps, Boc-amino acids and BOP reagent may be added in 5-fold excess in DMF solution followed by the same excess of DIEA. After a 2-hour reaction time, the completeness of each coupling may be monitored by the Kaiser test. See Kaiser, E.; Colescott, R.L.; Cook, P.I., *Anal Biochem.*, 34:595-598 (1970). Coupling of BocAsnOH may be repeated with 2.5 molar equivalent reagent to insure a high yield of the product.

After final removal of the Boc protecting group from the N terminus, the amino group may be liberated from its TFA salt by addition of DIEA (1.5 equivalents). The free amino group may cleave the peptide from the polymer support by intrachain aminolysis in DCM at room temperature. After a 24 hour reaction time, the product may be obtained from the solution phase by filtration. This crude product may be purified by silica gel chromatography (*e.g.*, 2 x 20 cm, eluent $CHCl_3$/MeOH/AcOH = 18/1/1).

Protecting groups of the peptide may be removed with TMSOTf in TFA in the presence of thioanisole, according to the procedure of Fujii, N. et al., *J. Chem. Soc., Chem. Commun.*, 274-275 (1987). Hydrolysis of the partly silylated product by $NH_4OH$ may be followed by gel permeation chromatography, for example, using Sephadex G-10 column (eluent, *e.g.*,: 2 M acetic acid in $H_2O$/MeOH, 4/1 [v/v]). Final purification may be carried out by RP-HPLC on, for example, a Vydac $C_{18}$ Proteins semi-preparative column eluted at, *e.g.*, 4 mL/min with a linear gradient of 25%-80% acetonitrile in 0.1% (v/v) TFA over 45 minutes.

In the above-described synthesis, one or more of the $N^{\alpha}$-Boc-amino acid starting materials may be purchased from chemical supply houses, for example, Sigma Chemical Company, PO Box 14508, St. Louis, Missouri 63178 (Sigma's "Peptides and Amino Acids" catalog provides a convenient listing) and Bachem, 6868 Nancy Ridge Dr., San Diego, CA 92121.

## EXAMPLE A2

The preparation of Loloatin B follows the synthesis of Loloatin A as described in Example A1, with the exception that BocTyr(2,6-$Cl_2$-Bzl)OH is replaced with BocTrp(Z)OH or other sidechain amine-protected L-tryptophan.

## EXAMPLE A3

The preparation of Loloatin C follows the synthesis of Loloatin B as described in Example A2, with the exception that BocPheOH is replaced with BocTrp(Z)OH or other sidechain amine-protected L-tryptophan.

## EXAMPLE A4

Solid phase peptide synthesis according to the method originally described by Merrifield, *J. Am Chem. Soc. 85*:2149-2154, 1963, the disclosure of which is hereby incorporated by reference, may be used to prepare the linear analogs of the cyclic decapeptides of the invention. Alternatively, solution synthesis may be used to prepare these linear peptide analogs. Generally, peptides may be elongated by deprotecting the α-amine of the C-terminal residue and coupling the next suitably protected amino acid through a peptide linkage using the methods described. This deprotection and coupling procedure is repeated until the desired sequence is obtained. This coupling can be performed with the constituent amino acids in stepwise fashion, or by condensation of fragments (two to several amino acids), or combination of both processes, or by solid phase peptide synthesis as stated above. Thereafter, the linear peptides may be cyclized by well known peptide cyclization techniques, to prepare the cyclic decapeptides of the invention.

When a solid phase synthetic approach is employed, the C-terminal carboxylic acid is attached to an insoluble carrier (usually polystyrene). These insoluble carriers contain a group which will react with the α-carboxyl group to form a bond which is stable to the elongation conditions but readily cleaved later. Examples of which include: chloro- or bromomethyl resin, hydroxymethyl resin, and aminomethyl resin. Many of these resins are commercially available with the desired C-terminal amino acid

already incorporated. Many of the suitably protected amino acids used in the present invention are also available commercially from Sigma Chemical Company and Bachem.

Alternatively, compounds of the invention can be synthesized using automated peptide synthesizing equipment. In addition to the foregoing, peptide synthesis are described in Stewart and Young, "Solid Phase Peptide Synthesis," 2nd ed., Pierce Chemical Co., Rockford, Ill. (1984); Gross Meienhofer, Udenfriend, Eds., "The Peptides: Analysis, Synthesis, Biology," Vol. 1, 2, 3, 5 and 9, Academic Press, New York, 1980-1987; Bodanszky, "Peptide Chemistry: A Practical Textbook," Springer-Verlag, New York (1988); and Bodanszky et al., "The Practice of Peptide Synthesis," Springer-Verlag, New York (1984), the disclosures of which are hereby incorporated by reference.

Coupling between two amino acids, an amino acid and a peptide, or two peptide fragments can be carried out using standard coupling procedures such as the azide method, mixed carbonic acid anhydride (isobutyl chloroformate) method, carbodiimide (dicyclohexylcarbodiimide, diisopropylcarbodiimide, or water-soluble carbodiimide) method, active ester (*p*-nitrophenyl ester, N-hydroxy-succinic imido ester) method, Woodward reagent K method, carbonyldiimidazole method, phosphorus reagents such as BOP-Cl, or oxidation-reduction methods. Some of these methods (especially the carbodiimide method) can be enhanced by adding 1-hydroxybenzotriazole. These coupling reactions can be performed in either solution (liquid phase) or solid phase.

The functional groups of the constituent amino acids must be protected during the coupling reactions to avoid formation of undesired bonds. The protecting groups that can be used are listed in Greene, "Protective Groups in Organic Chemistry," John Wiley & Sons, New York (1981) and "The Peptides: Analysis, Synthesis, Biology," Vol. 3, Academic Press, New York (1981), the disclosures of which are hereby incorporated by reference.

The α-carboxyl group of the C-terminal residue is usually protected by an ester that can be cleaved to give the carboxylic acid. Protecting groups which can be used include: (1) alkyl esters such as methyl and t-butyl, (2) aryl esters such as benzyl

and substituted benzyl, or (3) esters which can be cleaved by mild base treatment or mild reductive means such as trichloroethyl and phenacyl esters.

The α-amino group of each amino acid must be protected. Any protecting group known in the art can be used. Examples of which include: (1) acyl types such as formyl, trifluoroacetyl, phthalyl, and p-toluenesulfonyl; (2) aromatic carbamate types such as benzyloxycarbonyl (Cbz or Z) and substituted benzyloxycarbonyls, 1-(p-biphenyl)-1, -methylethoxy-carbonyl, and 9-fluorenylmethyloxycarbonyl (Fmoc); (3) aliphatic carbamate types such as *tert*-butyloxycarbonyl (Boc), ethoxycarbonyl, diisopropylmethoxycarbonyl, and allyloxycarbonyl; (4) cyclic alkyl carbamate types such as cyclopentyloxycarbonyl and adamantyloxycarbonyl; (5) alkyl types such as triphenylmethyl and benzyl; (6) trialkylsilane such as trimethylsilane; and (7) thiol containing types such as phenylthiocarbonyl and dithiasuccinoyl. The preferred α-amino protecting group is either Boc or Fmoc, preferably Fmoc. Many amino acid derivatives suitably protected for peptide synthesis are commercially available.

The α-amino protecting group is cleaved prior to the coupling of the next amino acid. When the Boc group is used, the methods of choice are trifluoroacetic acid, neat or in dichloromethane, or HCl in dioxane. The resulting ammonium salt is then neutralized either prior to the coupling or *in situ* with basic solutions such as aqueous buffers, or tertiary amines in dichloromethane or dimethylformamide. When the Fmoc group is used, the reagents of choice are piperidine or substituted piperidine in dimethylformamide, but any secondary amine or aqueous basic solutions can be used. The deprotection is carried out at a temperature between 0°C and room temperature.

Any of the amino acid bearing side chain functionalities must be protected during the preparation of the peptide using any of the above-described groups. Those skilled in the art will appreciate that the selection and use of appropriate protecting groups for these side chain functionalities depends upon the amino acid and presence of other protecting groups in the peptide. The selection of such protecting groups is important in that it must not be removed during the deprotection and coupling of the α-amino group. For example, when Boc is used as the α-amino protecting group, p-toluenesulfonyl (tosyl) moieties can be used to protect the amino side chain of Orn.

When Fmoc is chosen for the α-amine protection usually *tert*-butyl based protecting groups are acceptable. For instance, Boc can be used for ornithine.

Once the elongation of the peptide is completed all of the protecting groups are removed. When a solution phase synthesis is used, the protecting groups are removed in whatever manner is dictated by the choice of protecting groups. These procedures are well known to those skilled in the art.

When a solid phase synthesis is used, the peptide is cleaved from the resin usually simultaneously with the protecting group removal. When the Boc protection scheme is used in the synthesis, treatment with anhydrous HF containing additives such as dimethyl sulfide, anisole, thioanisole, or p-cresol at 0°C is the preferred method for cleaving the peptide from the resin. The cleavage of the peptide can also be accomplished by other acidic reagents such as trifluoromethanesulfonic acid/trifluoroacetic acid mixtures. If the Fmoc protection scheme is used, the N-terminal Fmoc group is cleaved with reagents described earlier. The other protecting groups and the peptide are cleaved from the resin using a solution of trifluoroacetic acid and various additives such as anisole, etc.

Subsequent to removal of the linear peptide from the resin and removal of any protecting groups as desirable, the linear peptide is cyclized using conventional procedures such as by treatment with triethylamine and diphenylphosphorylazide in dimethylformamide. Prior to purification of the crude cyclic peptide in the usual manner such as by use of chromatography, any remaining protecting and functional group precursors are removed or transformed into the desired group.

Example A4a. Synthesis of cyclo[L-Val-L-Orn-L-Leu-D-Tyr-L-Pro-L-Phe-D-Phe-L-Asn-L-Asp-L-Tyr] (Loloatin A)

Synthesis of the Linear Peptide

The linear analog of Loloatin A may be synthesized by standard solid phase methods using an Applied Biosystems (850 Lincoln Centre Dr., Foster City, California) 430A automated peptide synthesizer and protocols supplied by the manufacturer. Commercially available Boc-amino acids may be used with the following

side chain protection: Orn(Tos), Tyr(BrZ), Asp(Bzl). Commercially available Boc-Val-PAM resin (0.5 mmol, Applied Biosystems) may be deprotected with trifluoroacetic acid (2% anisole) and coupled in NMP with the HOBt esters of Boc-amino acids (4 equivalents). The HOBt esters of Boc-amino acids may be formed by the reaction of the Boc-amino acid with DCC and HOBt. Couplings may be carried out for 30 minutes and the resin subsequently washed with NMP and DCM. Any unreacted amine may be acylated with acetic anhydride. The deprotection and coupling may be repeated until complete assembly of the protected peptide resin is achieved. The linear peptide may be simultaneously deprotected and removed from the resin with anhydrous hydrogen fluoride (10 mL) at 0°C for 30 minutes in the presence of anisole (5%). The peptide may then be extracted with 50% acetic acid, water and aqueous acetonitrile, and lyophilized.

Cyclization of the Linear Peptide

The crude linear peptide prepared above may be dissolved in DMF (~5 µmol/ml) and cyclized with DPPA (5 equivalents) TEA to adjust the pH to 9. After completion of the reaction (4-48 hours), the solvent may be removed and the crude cyclic peptide lyophilized from acetonitrile/water. The cyclic peptide may then be desalted by gel filtration in, e.g., 70% acetic acid over a Spectragel GF05 column (2.5 x 55 cm), and purified by reversed phase preparative HPLC (Dynamax $C_{18}$, 21.4 x 250 mm, Rainin) using various gradients of 0.1% aqueous TFA and acetonitrile.

The purified peptide may then be characterized by Analytical HPLC (Vydac 218TP54, 4.6 x 250 mm), FAB-MS and amino acid analysis, and these characterizations compared to theoretical values when available.

EXAMPLE A5

The procedure of Example A4a may be modified to prepare Loloatin B. Thus, when Tyr(BrZ) is replaced with Trp(Z) or other amine-protected L-tryptophan, Loloatin B may be prepared.

## EXAMPLE A6

The procedure of Example A5 may be modified to prepare Loloatin C. Thus, when Phe is replaced with Trp(Z) or other amine-protected L-tryptophan, Loloatin C may be prepared.

## EXAMPLE A7

Each of Loloatin A, B and C may be isolated after fermentation of ATCC 55797 as described below.

The marine bacterial isolate MK-PNG-276A, tentatively identified as a *Bacillus laterosporus* by MIDI analysis of cellular fatty acids, was obtained from the tissues of an unidentified tube worm collected at -15 m off of Loloata Island, Papua, New Guinea. MK-PNG-276A has been deposited with the American Type Culture Collection as ATCC 55797.

MK-PNG-276A was cultured on trays of solid tryptic soy agar supplemental with NaCl to a concentration of 1%. Twenty-six 400 mL trays (9" x 15" x 1/4" deep agar) were cultured for five days after which the combined cells and agar were lyophilized. The lyophilization product, (61.5 g dry weight) was extracted with three 600 mL portions of methanol that were combined, filtered, and reduced *in vacuo* to give a brown/gray tar. The tar was dissolved in 750 mL of MeOH/$H_2O$ (1/4) and sequentially extracted with hexanes (3 x 250 mL) and EtOAc (3 x 250 mL). The combined EtOAc extracts were reduced *in vacuo* to give a taupe/brown crystalline solid (5.5 g). The EtOAc residue was then processed in batches. Size separation on an LH-20 Sephadex column with methanol eluant gave six fractions. The first and major fraction showed antibiotic activity against MRSA and *Enterococci* species. This fraction was then subjected to preparative reverse phrase column chromatography and RP HPLC using 9:1 methanol/water containing 0.1% TFA, to yield Loloatin A (relative retention time on HPLC=0.70, white powder 281 mg, 0.45% dry wt of cells), Loloatin B (relative retention time on HPLC=1.00, tan/white powder solid 1.87 g, 3.0% dry wt of cells) and Loloatin C (relative retention time on HPLC=0.66, tan/white powder 40 mg, 0.065% dry wt of cells).

Loloatin B (2) gave a (M+H) ion in the HRFABMS at m/z 1296.64232 appropriate for a molecular formula of $C_{67}H_{85}N_{13}O_{14}$ (ΔM+0.46 ppm). Detailed analysis of the $^1H$, $^{13}C$, COSY, HOHAHA, HMQC, HMBC and ROESY data for Loloatin B (2) identified the ten amino acids residues indicated in Table 1. Hydrolysis of 2 at 100°C with 6N HCl containing thioglycolic acid and examination of the pentafluropropionamide isopropyl ester derivatives of the liberated amino acids via chiral GC analysis confirmed the presence of L-valine, L-ornithine, L-leucine, D-tyrosine, L-proline, L-phenylalanine, D-phenylalanine, L-tryptophan and L-aspartic acid (from ASP and ASN). The ten identified amino acid residues accounted for all of the atoms in the molecular formula of (2), and 31 of the 32 sites of unsaturation demanded by the molecular formula. Thus, Loloatin B (2) had to be a monocyclic decapeptide.

The amino acid sequence in (2) was determined by analysis of HMBC and ROESY data. HRFABMS and MS/MS studies supported the amino acid sequence derived from the NMR data. The MS/MS data was consistent with initial cleavage of the ring at the TYR-CO/PRO-N bond to give a linear decapeptide that sequentially loses LEU-TYR (m/z 1019), ORN-LEU-TYR (m/z 905) and TRP-VAL-ORN-LEU-TYR (m/z 621). FABMS peaks at m/z 245 and 377 could be assigned to the protonated fragments PRO-PHE1 and PHE2-ASN-ASP, respectively.

The amino acid sequences for Loloatin A and C were determined in an analogous manner.

Table 1

$^1H$ NMR Data (500 MHz) for Loloatin B (2) (DMSO-$D_6$)

| Res. | | δ $^1H$ | Res. | | δ $^1H$ |
|---|---|---|---|---|---|
| VAL | NH | 7.52 (d,J=8.0) | PHE1 | $CH_2$ | 2.25 (m) |
| | αCH | 4.56 (m) | | *i*C | -- |
| | βCH | 2.01 (sept.J=7.0) | | *o*CH | 7.18 |
| | $\gamma CH_3$ | 0.93 (d) | | *m*CH | |
| | $\gamma CH_3$ | 0.93 (d) | | *p*CH | |
| | CO | -- | | CO | -- |
| ORN | NH | 8.88 (d,J=8.9) | PHE2 | NH | 9.05 (d,J=9.0) |
| | αCH | 5.27 (bm) | | αCH | 5.57 (bt) |
| | $\beta CH_2$ | 1.8 (m) | | $\beta CH_2$ | 2.75 (m) |
| | $\gamma CH_2$ | 1.7 (m) | | | 3.02 (m) |
| | $\delta CH_2$ | 2.8, 2.9 (m) | | *i*C | -- |

| Res. | | δ $^1$H | Res. | | δ $^1$H |
|---|---|---|---|---|---|
| | δNH | 7.45 (bs) | | *o*CH | 7.18 |
| | CO | – | | *m*CH | |
| | $CH_3$ | | | *p*CH | |
| | CO | | | CO | – |
| LEU | NH | 7.92 (bs) | ASN | NH | 9.03 (d,J=6.0) |
| | αCH | 4.55 (m) | | αCH | 4.49 (m) |
| | $\beta CH_2$ | 1.35 (m) | | $\beta CH_2$ | 3.37 (m) |
| | | 1.25 (m) | | | 3.0 (m) |
| | γCH | 1.5 (m) | | CO | – |
| | $\delta CH_3$ | 0.93 (d) | | $NH_2$ | 8.04 (bs) |
| | $\delta CH_3$ | 0.93 (d) | | | 7.45 |
| | CO | – | | CO | – |
| TYR | NH | 9.21 (s) | ASP | NH | 8.32 (d,J=4.2) |
| | αCH | 4.22 (m) | | αCH | 4.26 (m) |
| | $\beta CH_2$ | 2.70 (m) | | $\beta CH_2$ | 2.35 (m) |
| | | 2.81 (m) | | | 2.25 (m) |
| | *i*C | – | | γCO | – |
| | *o*CH | 6.98 (d,J=8.6) | | $CH_3$ | |
| | *m*CH | 6.61 (d,J=8.6) | | CO | – |
| | *p*COH | not observed | TRP | NH | 8.63 (d,J=9.8) |
| | CO | – | | αCH | 4.5 (m) |
| PRO | N | – | | $\beta CH_2$ | 3.15 (m) |
| | αCH | 4.07 (d,J=7.6) | | C | – |
| | $\beta CH_2$ | 1.43 (m) | | C | – |
| | | 1.25 (m) | | CH | 7.5 |
| | $\gamma CH_2$ | 1.07 (m) | | CH | 7.0 |
| | | 0.411 (m) | | CH | 7.03 (m) |
| | $\delta CH_2$ | 2.20 (m) | | CH | 7.32 (J=8.0) |
| | | 3.30 (m) | | C | – |
| | CO | – | | NH | 10.81 (bs) |
| PHE1 | NH | 7.23 (d,J=9.5) | | CH | 7.02 (d) |
| | αCH | 4.5 (m) | | CO | – |

## B. ACTIVITY EXAMPLES

### EXAMPLE B1

In a standard liquid dilution antimicrobial assay described below, Loloatin B was found to be selectively antimicrobial, with the minimum inhibitory concentrations listed in Table 2 below. Antimicrobial activity was determined by macrobroth dilution antimicrobial susceptibility testing. A solution of Loloatin B was prepared in tryptic soy broth. Initially, a 100 μg/ml solution of the peptide was tested. If inhibition of a target

microorganism was detected, serial two-fold dilutions (in broth) of the Loloatin B solutions were tested to determine the minimal inhibitory concentration (MIC) of Loloatin B for each target organism. Target organisms tested are identified in Table 2. Turbidity standardized suspensions of each target organism were prepared according to accepted protocols using a 0.5 McFarland turbidity standard, and these standardized suspensions were used to inoculate a tube containing Loloatin B. Activity of Loloatin B was indicated by lack of growth (turbidity) of one or more of the target organisms.

See Woods, G.L. et al., "Antibacterial susceptibility tests: dilution and disk diffusion methods, *Manual of Clinical Microbiology* (6th Ed.), Murray, Baron, Pfaller, Tenover and Yolken (Eds.), ASM Press, Washington DC, 1995, pp. 1327-1341.

Table 2

Minimum Inhibitory Concentrations of Loloatin B
Against a Panel of Human Pathogens

| | |
|---|---|
| Methicillin resistant *Staphylococcus aureus* | < 2 µg/mL |
| Vancomycin resistant *Enterococcus faecalis* | < 2 µg/mL |
| Penicillin resistant *Streptococcus pneumoniae* | < 2 µg/mL |
| *Candida albicans* | > 100 µg/mL |
| *Pseudomonas aeruginosa* | > 100 µg/mL |
| *Enterobacter cloacae* | > 100 µg/mL |
| *Xanthomonas maltophilia* | > 100 µg/mL |
| *Escherichia coli* | > 100 µg/mL |

EXAMPLE B2

Antimicrobial activity was determined by macrobroth dilution antimicrobial susceptibility testing. Solutions of the cyclic decapeptides (Loloatin A, B and C) and a control (vancomycin) were prepared in tryptic soy broth. Initially, a 100 µg/ml solution of each compound was tested. When inhibition of target microorganism was detected, serial two-fold dilutions of the compound in broth were tested to determine the minimal inhibitory concentration (MIC) of each compound for each target organisms. Target organisms tested are listed below in Table 3, and included methicillin resistant *Staphylococcus aureus*, vancomycin resistant *Enterococcus sp.*, *Escherichia*

*coli*, multiply drug resistant *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Candida albicans*. Turbidity standardized suspensi ns of each target organism were prepared according to accepted protocols using a 0.5 McFarland turbidity standard, and these standardized suspensions were used to inoculate the tubes containing the compounds. Activity of a compound was indicated by lack of growth (turbidity) of one or more of the target organisms.

See Woods, G.L. and Washington, J.A., "Antibacterial susceptibility tests: dilution and disk diffusion methods, *Manual of Clinical Microbiology* (6th Ed.), Murray, Baron, Pfaller, Tenover and Yolken (Eds.), ASM Press, Washington DC, 1995, pp. 1327-1341.

The data of Table 3 demonstrates that Loloatin C has excellent activity against *Escherichia coli*, a gram negative rod bacteria.

Table 3

Antibacterial Activity Of The Loloatins
(Broth Dilution MICs in µG/mL)

| | Loloatin A | Loloatin B | Loloatin C | Vanco-mycin |
|---|---|---|---|---|
| *S. aureus (MRSA)* | 2-4 | 2-4 | 0.5-1 | |
| *Enterococcus faecalis (ATCC51299)* | 2-4 | 2-4 | 2-4 | 20 |
| *Enterococcus faecalis (13242)* | 2-4 | 2-4 | 1-2 | >100 |
| *Enterococcus faecium (F4641)* | 2-4 | 2-4 | 2 | 6 |
| *Enterococcus faecium (19007)* | 2-4 | 2-4 | 2 | >100 |
| *Enterococcus gallinarum* | 2-4 | 2-4 | 1-2 | 6 |
| *Group A Streptococcus (19615)* | <0.25 | <0.25 | <0.25 | |
| *Group B Streptococcus (12401)* | <1 | <1 | <0.25 | |
| *Candida albicans* | 16 | 16 | 16 | |
| *Pseudomonas aeruginosa* | >100 | >100 | >100 | |
| *Enterobacter cloacae* | >100 | >100 | >100 | |
| *Stenotrophomonas maltophilia* | >100 | >100 | >100 | |
| *Escherichia coli* | >100 | >100 | 1-2 | |

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually incorporated by reference.

From the foregoing, it will be evident that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention.

## Claims

What is claimed is:

1. An isolated compound selected from the group consisting of:
cyclo [L-Val-L-Orn-L-Leu-D-Tyr-L-Pro-L-Phe-D-Phe-L-Asn-L-Asp-L-Tyr];
cyclo [L-Val-L-Orn-L-Leu-D-Tyr-L-Pro-L-Phe-D-Phe-L-Asn-L-Asp-L-Trp];
and
cyclo [L-Val-L-Orn-L-Leu-D-Tyr-L-Pro-L-Trp-D-Phe-L-Asn-L-Asp-L-Trp].

2. A compound of claim 1 having the formula:
cyclo [L-Val-L-Orn-L-Leu-D-Tyr-L-Pro-L-Phe-D-Phe-L-Asn-L-Asp-L-Tyr].

3. A compound of claim 1 having the formula:
cyclo [L-Val-L-Orn-L-Leu-D-Tyr-L-Pro-L-Phe-D-Phe-L-Asn-L-Asp-L-Trp].

4. A compound of claim 1 having the formula:
cyclo [L-Val-L-Orn-L-Leu-D-Tyr-L-Pro-L-Trp-D-Phe-L-Asn-L-Asp-L-Trp].

5. A pharmaceutical composition comprising a compound of any of claims 1-4 and a pharmaceutically acceptable carrier.

6. A method for the treatment of a patient afflicted with a bacterial infection comprising the administration to said patient of a therapeutically effective amount of a compound of any of claims 1-4.

# CYCLIC DECAPEPTIDE ANTIBIOTICS

## Abstract of the Disclosure

Three cyclic decapeptides having antibiotic activity are disclosed. The decapeptides are active against both gram positive and gram negative bacteria.